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BIOCOMPATIBILITY TESTING OF POLYMERS: 'IN VITRO' STUDIES WITH '--ETC(U)
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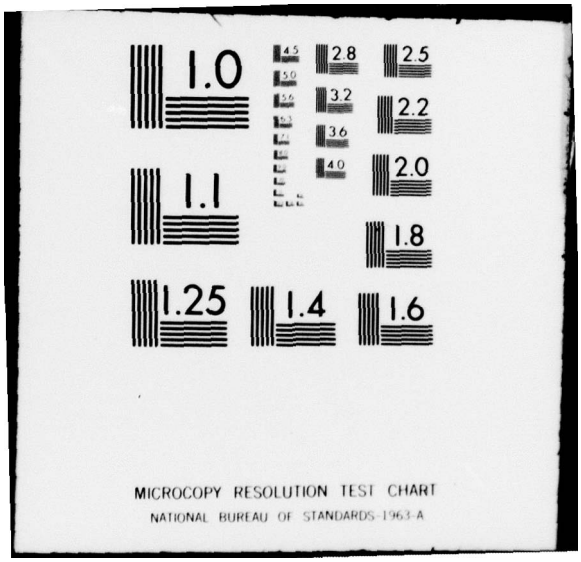
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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
See Supplementary Notes		

4. TITLE (and Subtitle)
Biocompatibility Testing of Polymers: In vitro Studies with in vivo correlation

5. TYPE OF REPORT & PERIOD COVERED
Publication in Journal

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)
Robert M. Rice*; Andrew F. Hegyeli;** Stuart J. Gourlay;*** Clarence W. R. Wade, James G. Dillon;*** Howard Jaffe;***** and R. K. Kulkarni

8. CONTRACT OR GRANT NUMBER(s)
14 p.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
US Army Medical Bioengineering Research & Development Laboratory, Fort Detrick, Frederick, Maryland 21701

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS

11. CONTROLLING OFFICE NAME AND ADDRESS
*Present address: USAMRIID, Ft. Detrick, Frederick, Md.21701. Author to whom all correspondence should be addressed.

12. REPORT DATE
1 April 1978

13. NUMBER OF PAGES
12

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)
21 apr 77

15. SECURITY CLASS. (of this report)
UNCLASSIFIED

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)
Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES
J. Biomedical Materials Research 12, 43 - 54 (1978

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
Biocompatibility testing; Polymers; in vitro; in vivo; Poly(lactic acid); Poly(d-cyanoacrylates); Poly(e-caprolactone); methods comparison Poly(glycolic acid)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
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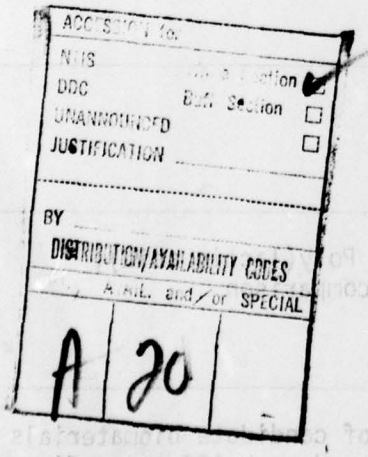

Biocompatibility Testing of Polymers: *In vitro* Studies with *in vivo* Correlation

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Summary

An *in vitro* method has been developed for screening of candidate biomaterials in an early phase of their development. The test is based on L-929 mouse fibroblast cultures and their response to powdered polymer samples. It applies microscopic observation for the detection of morphological changes, uses dye exclusion testing for cell viability determination, and utilizes estimation of population doublings as an end point. The test is shown to be reliable and reproducible and is compared to *in vivo* implantation studies in rats, previously reported.

INTRODUCTION

In the development of biomaterials for eventual use in humans, thorough testing for biocompatibility in experimental animals before the initiation of human studies is necessary. If unsuitable materials are not eliminated by screening tests at an early phase in their development, extensive sums of money and valuable time could be

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Journal of Biomedical Materials Research, Vol. 12, 43–54 (1978)

0021-9304/78/0012-0043$01.00

wasted on the development of materials to find that the final stage of animal testing shows the material not fit for human use.

Tissue culture test systems for screening of candidate materials for biocompatibility are increasing in popularity, and the addition of *in vitro* methods of biocompatibility testing in standard *in vivo* methods can increase test accuracy because of their greater sensitivity.[1–5] It cannot be expected that a single *in vivo* or *in vitro* test will always predict the response in humans to the material; however, the use of a series of tests will increase the probability of accurately classifying a material as biocompatible.[6] In the development of polymeric materials for tissue adhesives, surgical sutures and other surgical repair materials, the U.S. Army Medical Bioengineering Research and Development Laboratory found *in vitro* testing valuable when used as an adjunct to *in vivo* testing for degradation studies of biomaterials.[7–11] The purpose of this work was to develop a tissue culture screening method to predict the biocompatibility of polymers during early phases of development.

Three principal tissue culture methods have been used for testing of biomaterials: 1) agar overlay technique;[2,3,12] 2) direct contact of cells with the surface of the biomaterial;[13,14] and 3) radioactive tracers.[15,16] These methods are applicable to fabricated prototype materials. In using a tissue culture method as a screening technique in the development of polymeric materials, it would be better to have a technique to test materials in particulate form. Of the three methods mentioned above, only the radioactive tracer technique could be utilized. Whenever radioactive materials are used, special equipment and laboratory safety techniques are necessary. Therefore, a method whereby cellular functions were monitored by other means was developed.[21]

Microscopic observation, dye exclusion, and population doubling (P.D.) determinations were the methods utilized to assess the effect of the polymeric material on the following cellular functions: attachment, viability, and division. These parameters were used to develop a tissue culture test for application as a screening test in conjunction with a short term *in vivo* implantation study[18] to predict the biocompatibility of a material. This study was conducted blindly with powdered polymers in conjunction with the *in vivo* evaluation of the same materials[18] and the *in vitro* and *in vivo* results were correlated.

MATERIALS AND METHODS

Table I identifies the polymers evaluated as well as their source. Polymers were tested in powdered form, and approximately 10 mg of each polymer was placed in a #3 gelatin capsule (Parke, Davis & Co., Detroit, Mich.). Capsules were individually heat-sealed in plastic envelopes and sterilized with γ-radiation (1.5 Mrad) from a ^{60}Co source.

Sterilized capsules were opened aseptically and the contents of each were placed into a Falcon plastic tissue culture flask with a 25 cm^2 growing surface. Five replicates of each polymer were evalu-

TABLE I
Polymers Evaluated and Their Sources[a]

Polymer Tested	Abbreviation	Source
Poly(acrylic acid)	PAA	Polysciences, Inc.
Poly(1,2-isopropylidene)glycerol cyanoacrylate (high M.W.)	PIGC-h	In-House
Poly(propiolactone) (high M.W.)	PPL-h	In-House
Orlon	O	In-House
Poly[L(+)lactic acid]	PLA-l	In-House
Poly(propiolactone)	PPL	In-House
Poly(1,2-isopropylidene)glycerol cyanoacrylate (low M.W.)	PIGC-l	In-House
Poly[methyl L(+)lactyl 2-cyano-acrylate]	PMLC	In-House
Nylon 66	N	DuPont, Wilmington, Del.
AN/PL (crude)	AN/PL	In-House
AN/PL (extracted)	AN/PL-e	In-House
Poly(acrylamide)	PA	In-House
Poly(caprolactone)	PCL	In-House
Poly(methyl methacrylate)	PMMA	L. O. Caulk Co., Milford, Del.
Poly(ethylene oxide) lot #1120	PEO	Union Carbide
Teflon #10086	T	DuPont, Wilmington, Del.
Poly(ethylmethylenemalonate)	PEMM	In-House
Poly(glycolic acid)	PGA	In-House
Poly(methyl 2-cyanoacrylate)	PMC	In-House
Poly(isobutyl-2-cyanoacrylate)	PIB	In-House

[a] In-house materials were synthesized at the U.S. Army Medical Bioengineering Research and Development Laboratory, Ft. Detrick, Md. 21701.

ated. Cell inocula containing between 200,000 and 300,000 viable L-929 transformed mouse fibroblast cells (Sanford, Earle and Likely) as previously described[21] were placed in the tissue culture flasks with 5 ml of Minimum Essential Medium (MEM) with 0.1 ml of 1 *M* Hepes Buffer. Flasks were incubated at 37°C for 48 hr and observed at least twice during the incubation period for changes in their normal growth pattern. At the end of the incubation period, cells were harvested, counted and the population doublings determined by the following formula similar to that described by Massie, Baird, and Samis[22]:

$$\text{P.D.} = \ln (N/N_0)/0.693$$

where N = number of cells after 48 hr and N_0 = number of viable cells in the inoculum. Percent cell viability was determined by exposing cells to trypan blue and then counting both the total cells and the number viable microscopically using a hemocytometer. Total cell counts were determined by modification of a turbidity technique described by Grinwell and Spere.[23] Cell aliquots were suspended in calcium–magnesium-free phosphate-buffered saline[24] in a cuvette, and an absorbance reading was taken on a Bausch & Lomb Spectronic 20 at 620 mμ. The absorbance was converted to a total cell count from a previously calibrated curve. The total number of viable cells was obtained by multiplying the percentage of viable cells times the total count.

Several powdered polymers were blindly evaluated along with three controls. All the polymers could not be simultaneously evaluated; however, each time any of the polymers were tested, both a control (no polymer added) and a known biocompatible polymer (PGA) were included with each test group. The data obtained for each test group were evaluated by an analysis of variance.[39] The mean P.D. values of the test polymers were compared to those of the control and PGA to determine if there was a significant difference ($\alpha = 0.05$).[40] Depending on the statistical evaluation, each individual compound was placed in one of three cytotoxicity classes:

I. Noncytotoxic; mean P.D. was not significantly less than the control and PGA values.

II. Moderately cytotoxic; mean P.D. was significantly less than both the control and PGA cultures.

III. Cytotoxic; all the cells were killed when exposed to the polymer.

The correlation between the *in vitro* and *in vivo*[18] results was determined by the Spearman's rank correlation method.[39]

RESULTS

Results of the *in vitro* evaluation of the various polymers are summarized in Tables II and III. Microscopic observation are tabulated in Table II and mean P.D.'s for each polymer are listed in Table III with their respective control and PGA cultures. Statistical significance of differences from respective control and PGA values are indicated in Table III along with toxicity groups for all polymers.

TABLE II

Polymer Cytotoxicity: Microscopic Observations[a]

Polymer	Observation Nos.	Observations
PPL-h	1	1. Normal cell attachment with normal growing cells throughout 48 hr test period.
PPL-h	1	
PPL	1	
N	1	2. A few cells attached initially.
T	1	3. The few cells that attached appeared to grow normally.
PLA-l	1	
PEO	1	4. Few cells attached after 48 hr.
PA	1	5. All cells in medium dead.
PCL	1	6. All cells attached to growing surface dead after 48 hr.
O	1	
AN/PL	1	7. Cell clumping.
AN/PL	2, 3	8. No cells attached to growing surface.
AN/PL-e	2, 3	
PMMA	1, 11	9. Had a sudden pH change to 5.2.
PMLC	7, 8, 10	
PIGC-h	4	10. Had several variable cells in medium after 48 hr.
PIGC-l	2, 5, 6, 7	
PICC-l	2, 5, 6, 7	11. Polymer adhered to cells.
PAA	5, 8, 9	
PMC	5, 7, 8	
PEMM	2, 5, 6, 7	
(PIBC)	1	
(PMC)	5, 7, 8	
(PGA)	1	
Controls	1	

[a] Materials in parentheses were evaluated as knowns; all others were tested as unknowns.

TABLE III

Results of *in vitro* Experimentation

Polymer	Mean P.D.	Mean P.D.[a] of Respective		Significantly[a] Less Than		Toxicity Class
		Control	PGA	Control	PGA	
PPL-h	2.7	2.7	2.0	No	No	I
PPL-h	2.1	2.2	2.6	No	No	I
PPL	2.1	1.8	1.4	No	No	I
N	1.9	2.0	1.9	No	No	I
T	2.1	2.2	2.2	No	No	I
PLA-l	1.6	1.8	1.4	No	No	I
PEO	2.1	2.2	2.2	No	No	I
PA	2.0	2.3	2.1	Yes	No	I
PCL	2.1	2.3	2.1	Yes	No	I
O	2.1	2.7	2.0	Yes	No	I
AN/PL	2.1	1.9	1.6	Yes	Yes	II
AN/PL	0.6	1.5	1.4	Yes	Yes	II
AN–PL-e	1.2	2.3	2.1	Yes	Yes	II
PMMA	—[b]	2.2	2.2	Yes	Yes	II
PMLC	—[c]	2.3	2.1	Yes	Yes	II
PIGC-h	0.4	1.5	1.4	Yes	Yes	II
PIGC-e	0.9[d]	2.3	2.1	Yes	Yes	III
PIGC-l	—[e]	2.3	2.1	Yes	Yes	III
PAA	—[e]	1.9	1.7	Yes	Yes	III
PMC	—[e]	2.3	2.1	Yes	Yes	III
PEMM	—[e]	2.2	2.2	Yes	Yes	III
PIBC[f]	2.0	2.4	2.3	Yes	Yes	II
PMC[f]	—[e]	2.4	2.3	Yes	Yes	III
PGA[f]	2.0[g]	2.0	2.0	No	—	I

[a] Significant differences were determined by analysis of variance and least significant difference testing ($\alpha = 0.05$).

[b] The polymer adhered to the cells and the P.D. could not be evaluated by the optical density technique. By microscopic evaluation, the population was determined to be significantly less than the control and PGA P.D.

[c] After 48 hr no cells were on growing surface; however, approximately 70% of cells in medium were viable. The P.D. could not be calculated the same way and since there was evidence of some cytotoxicity, it was placed in Class II.

[d] Had a P.D. of 0.9; however, all the cells were dead that were on the growing surface. Therefore, it was placed in Class III.

[e] All cells were killed and became detached from the growing surface. The P.D. had no meaning.

[f] Polymer that was evaluated as a known while all others were evaluated blindly.

TABLE IV
Comparison of Polymer Toxicity from Literature to Experimental Toxicity

Polymer	Toxicity from Literature		References	Experimental Toxicity Class	
	in vitro	*in vivo*		*in vitro*	*in vivo*[a]
N	N-M	N-M	1,25–27	I (N)	I (N)
T	N-M	N-M	25,26,28	I (N)	II (M)
PLA-l	N	N	10,29	I (N)	I (N)
PEO	N-M	N	26	I (N)	I (N)
PA		N	30	I (N)	I (N)
O		M	1	I (N)	II (M)
PMMA	M	N-M	27,31,37	II (M)	II (M)
PAA	N	N	32	III (T)	III (T)
PMC	T	T	20,28,33–35	III (T)	III (T)
PIBC	M-5	M	7,20,34,36	II (M)	II (M)
PGA	N	N	21,28	I (N)	I (N)

[a] Results of *in vivo* experiments of Gourlay et al.[18]

Note: N – *in vitro* little or no growth inhibition, *in vivo* slight or no tissue reaction; M – *in vitro*—moderate growth inhibition, *in vivo*—moderate tissue reaction; T – *in vitro*—extensive growth inhibition, *in vivo*—severe tissue reaction

Table IV lists results of polymer toxicity reported in the literature for many of the polymers that were evaluated in this study. It also lists the results of *in vitro* and *in vivo* experiments for these polymers from this study and a previous study.[18]

Table V lists the *in vitro* and *in vivo*[18] toxicity classification for additional laboratory polymers evaluated by both methods for which comparative values could not be found in the literature.

The Spearman rank correlation coefficient for the *in vivo* versus *in vitro* data was 0.68 and was significantly different from 0 ($\alpha = 0.05$). Table VI represents a scatter diagram of the data.

DISCUSSION

Observations, both gross and microscopic, made over the 48 hr of the experiment were an important aspect of this method because

[g] PGA was used as a negative control in each test group. The mean P.D.'s of the PGA and control for the various test groups were compared by a paired *t*-test and found not to be significantly different ($\alpha = 0.05$).

TABLE V
Experimental Toxicity of Laboratory Polymers

Polymer	*in vitro* Toxicity Group	*in vivo* Toxicity Group
PPL-h	I	II
PPL-h	I	II
PPL	I	III
PCL	I	II
AN/PL	II	II
AN/PL-e	II	III
PMLC	II	II
PIGC-h	II	III
PIGC-l	III	II
PIGC-l	III	II
PEMM	III	II

TABLE VI
Scatter Diagram of *in vitro* Toxicity Class vs. *in vivo* Toxicity Class[a]

	in vivo		
in vitro	I	II	III
I	5	5	1
II		4	2
III		3	2

[a] Numbers in the columns represent the number of samples that fell into each category.

they allowed detection of deviations from expected cell behavior and gave a more complete picture on the interaction between the cells and polymer. For instance, the polymer PAA has been reported to be nontoxic.[32] However, from Table II it can be seen that based on the tests conducted in this study, it falls into the cytotoxic class. It was noted (Table II) that this particular polymer inducted a sudden drop in pH to 5.2 which appeared to be responsible for killing all the cells. Peters et al.[32] found no growth inhibition when they used 0.8 mg of PAA; in this experiment we used 10 mg. Therefore, from these observations of the change in pH and the previous observations that the polymer is nontoxic, it is assumed

that the solubility and acidity of the polymer overwhelmed the buffering capacity of the tissue culture system.

PIGC-1 was one of the duplicate samples in the evaluation. In one of the PIGC-1 samples, the dead cells did not become detached from the growing surface; thus, the test would have given erroneous results if the classification had been based on the P.D. value alone since the final cell count was not corrected for dead cells. However, on microscopic observation of the flasks prior to the termination of the experiment, it was noted that the cells still attached to the growing surface were not normal and staining with trypan blue showed that all these cells were dead. On the basis of this observation, the PIGC-l was placed in the cytotoxic group.

One other polymer (PMLC) was observed to interact with the cells differently than the others. PMLC prevented the cells from attaching to the growing surface and yet did not kill all of the cells. At the end of 48 hr, approximately 70% of the cells in the medium were viable. A P.D. couldn't be determined by the usual method and since 30% of the cells were dead, the polymer was placed in the moderately toxic classification. The above three examples show that microscopic observations during the experiment were an important aspect and were crucial to the outcome of this experiment.

Powdered PGA was chosen as a negative control because in previous experience[21] it was shown to be nontoxic to tissue culture cells. Due to physical limitations of facilities, all of these polymers could not be simultaneously evaluated. Therefore, the large group of polymers was broken up into subgroups and evaluated at different times. When each group of polymers was evaluated, PGA was included as a negative control along with a control culture containing no polymer. In some experiments, the P.D. of the control and PGA differed. However, over the course of the entire series of experiments the mean P.D.'s of PGA and control did not differ significantly as determined by a paired t-test at $\alpha = 0.05$. Since PGA was considered nontoxic, it was assumed that any polymer which was not significantly different by analysis of variance at $\alpha = 0.05$ from its respective control and PGA values was also nontoxic.

All of the polymers listed in Table III were evaluated blindly except PIBC, PMC, and PGA, as indicated by footnote f for this table. There was no P.D. for polymers EMM, PMCM, PAA and PIGC-l (one of the duplicate samples) since they killed all the cells

and caused them to detach from the growing surface. A mean P.D. for PMMA could not be calculated on the basis of these experiments because the polymer adhered to the cell surface and could not be washed off. Since this would lead to an erroneous P.D. determination by increasing the absorbance reading, PMMA was classified as a moderately cytotoxic compound on the basis of microscopic examination of the culture flasks, which indicated that the cell population size for PMMA was consistently less than the control and PGA cultures.

Documentation of the reproducibility of these experimental methods is provided by the finding that the toxicity classifications for each of the duplicate polymers (PPL-h, AN/PL, and PIGC-l) were identical (Table III). There is good agreement generally between the results of this experiment and results reported by other investigators (Table IV). It is felt that this method is reliable.

The correlation between *in vitro* and *in vivo* data (Tables IV and V) is significant ($\alpha = 0.05$) with a Spearman's rank correlation coefficient of 0.66. Table VI displays some of the data contained in Tables IV and V in a type of scattergram. If perfect correlation were to exist between the *in vitro* and *in vivo* data, the numbers in Table VI would have appeared only in blocks I-I, II-II, and III-III. Even though the correlation coefficient was significant, it can be seen from Table VI that the correlation was not perfect. Leininger[1] has stated that no compound which is nontoxic *in vitro* will be toxic by intermuscular implantation. In these studies, one compound (PPL) was found to be nontoxic in the *in vitro* test and toxic in the *in vivo* evaluation. Spangberg and Langeland[5] state: "a material which is toxic *in vitro* can always be expected to cause tissue irritation. Low toxicity *in vitro*, however, is not equal to low tissue irritation." The data presented in this paper better support this latter statement. All of the compounds that were moderately toxic or toxic in the tissue culture evaluation (Table VI) produced a moderate or extensive tissue reaction in the rat intermuscular implantation studies.[18]

In conclusion, the *in vitro* test described in this paper and the *in vivo* test, described previously, by themselves did not completely agree with reported results for the compounds tested. When the two tests were used together, they were in closer agreement with the reported results. Therefore, it is suggested that in toxicity testing of materials, one should not rely on just one test alone[38] but should

utilize a battery of tests. The methods described in this and a previous paper[18] are screening tests aimed at eliminating cytotoxic materials early in their development phase. They cannot replace nor are they intended to replace the long-term animal studies which are required prior to human usage of a material.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or Department of Defense.

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Received April 21, 1977